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A New Species of the Genus *Crenubiotus* (Tardigrada: Eutardigrada: Adorybiotidae) from Salt Spring Island, Strait of Georgia, British Columbia (Canada)

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Original article

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Currently, the recently erected genus *Crenubiotus* (Adorybiotidae, Macrobiotoidea) includes only three species, all of which are characterised by dentate lunulae and cuticular tubercules organised in the band in the dorso-caudal part of the body. By means of integrative taxonomy, we describe a fourth species of the genus: *Crenubiotus salishani* sp. nov., from Salt Spring Island in British Columbia, Canada. The new species has been found in the moss growing on rock and differs from the other species in the genus due to the presence of a median anterior mucrone in the third band of the oral cavity armature (OCA) and by the presence of evident thickenings on the eggshell connecting the neighbouring processes. This finding highlights the importance of continuing to study tardigrade biodiversity, even in already explored areas, and how an integrative approach is fundamental to achieving a reliable measure of biodiversity.

Key words: Southern Gulf Islands, Salish Sea, egg ornamentation, tardigrades, integrative taxonomy, confocal microscopy.

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Tardigrades from British Columbia, Canada have been recorded since 1908, but knowledge of the baseline data and distribution ranges in the region (and of the Canadian tardigrade fauna as a whole) remains sparse. For more than 80 years after Richter's first mention of tardigrades from Vancouver in 1908 and Murray's collections in 1910, there were only fourteen published accounts of the Canadian tardigrade fauna (KATHMAN 1990); furthermore, there were no further studies on the tardigrades from British Columbia until that of KATHMAN & DASTYCH in 1990. More recently, VECCHI *et al*. (2022) described a new species of *Sisubiotus* from this biogeographic region, highlighting the importance of continued sampling and the potential for biodiversity discoveries here and elsewhere, even in areas considered to be already explored.

The genus *Crenubiotus* was recently erected (LISI *et al.* 2020) and currently includes only three species: *C. crenulatus* (RICHTERS 1904); the type species – *C. revelator* (LISI, LONDOÑO & QUIROGA 2020); and *C. ruhesteini* (GUIDETTI, SCHILL, GIOVANNINI, MASSA, GOLDONI, EBEL, FÖRSCHLER, REBECCHI & CESARI 2021). The genus is easily recognisable by its characteristic big trapezoidal lunules with long and narrow teeth, and by the flat cuticular tubercles forming a band in the dorso-caudal region of the animal's body (LISI *et al.* 2020). *Crenubiotus* is placed within the family Adorybiotidae, together with *Adorybiotus* (STEC *et al.* 2020a). The two genera share similar den-

tate lunulae and dorso-caudal cuticular tubercules, but they differ in their buccal apparatus: there is a very prominent dorsal hook and thick buccal tube walls in *Adorybiotus*; with no dorsal hook and thinner buccal tube walls in *Crenubiotus*. The eggs of the two genera are also very different, with dichotomous branched processes in *Adorybiotus* and simple conical processes occurring in *Crenubiotus*. An examination of a moss sample from Salt Spring Island in British Columbia, Canada, revealed the animals and eggs of a new, previously undescribed *Crenubiotus* species. Here, we will describe the new species known as *Crenubiotus salishani* sp. nov. with light microscopy and confocal microscopy (CF), as well as providing DNA sequences of the molecular markers commonly used in tardigrade taxonomy (18S and 28S rRNA, COI and ITS2). Finally, we will determine the phylogenetic position of the new species.

Materials and Methods

Samples and specimens

A moss sample (Field collection code JYU.S1916, RBCM.HC2021-7, 24/06/2021) growing on rock was collected on 24/06/2021 by one of the authors (HC) from Ruckle Provincial Park (48°46′16.8″N 123°22′39.7″W, Salt Spring Island in British Columbia, Canada, at an elevation of 23 m). Salt Spring Island is one of the Southern Gulf Islands in the Strait of Georgia, situated between mainland British Columbia and Vancouver Island. The sample was air-dried and kept desiccated until the analysis. The sample was examined for the presence of tardigrades using the sieving protocol developed by DASTYCH (1980). The animals and eggs were split into several groups for specific taxonomic analyses: a morphological analysis in Phase Contrast Microscopy (PCM) (4 animals, 14 eggs); and DNA (2 animals) sequencing. One of the eggs mounted for the PCM was also imaged with Confocal Microscopy (CF). Two additional *Adorybiotus* sp. individuals (Sample code JYU.S1911 GSB-Loc.5; moss on rock; S1911; 51°38′54.1″N 128°08′38″W, Calvert Island, Canada; Gillian Sadlier-Brown leg., 29/06/2021) were used for DNA sequencing to improve the phylogenetic reconstruction.

Microscopy and imaging

The specimens for light microscopy were mounted on microscope slides in a small drop (~200 mg) of Hoyer's medium, secured with a cover slip (22x22 mm) and dried at 60°C for a week. The slides were examined under an Olympus BX53 light microscope with PCM, associated with an Olympus DP74 digital camera. All the figures were assembled with FigureJ (MUTTERER & ZINCK 2013). For the structures where a focus could not be satisfactorily achieved in a single light microscope photograph, a stack of 2-4 images were taken with an equidistance of ca. 0.2 µm and were manually assembled into a single deep-focus image in GIMP 2.10 (GIMP DEVELOPMENT TEAM 2019). A single egg mounted in Hoyer's medium was imaged by exploiting its autofluorescence for a 3D reconstruction with a LeicaSP8 Falcon confocal microscope, which was available at the Department of Biological and Environmental Sciences (University of Jyväskylä), with an excitation wavelength of 551 nm and an emission of 558-665 nm. 85 equidistant slices were imaged in a section of 25µm. The 3D reconstruction from the CF images was done with the VolumeViewer plugin for Fiji (SCHINDELIN *et al.* 2012).

Morphometrics and morphological nomenclature

All measurements are given in micrometres (µm). The structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the caudal extent of the body, excluding the hind legs. The length of the buccal tube and the level of the insertion point of the stylet supports were measured according to PILATO (1981). The *pt* index is the ratio of the length of a given structure to the length of the buccal tube (PILATO 1981). The terminology used to describe the oral cavity armature, as well as the measurements of the buccal tube widths, heights of the claws and the eggs followed that of KACZMAREK & MICHALCZYK (2017). The description of the cuticular bars and muscle attachments above the claws followed that of KIOSYA *et al.* (2021). The morphometric data was handled using the 'Parachela' ver. 1.7 template, available from the Tardigrada Register (MICHALCZYK & KACZMAREK 2013). The raw morphometric data is provided as Supplementary Materials (SM.01). The tardigrade taxonomy followed BERTOLANI *et al.* (2014), STEC *et al.* (2020a), STEC *et al.* (2021) and GUIDETTI *et al.* (2021).

Genotyping

DNA was extracted from individual animals following the Chelex® 100 resin extraction method (BioRad) by CASQUET *et al.* (2012) with the modifications described in detail in STEC *et al.* (2020b). Each specimen was mounted in water and examined under a light microscope, prior to the DNA extraction. We sequenced four DNA fragments: three nuclear (18S rRNA, 28S rRNA, ITS2) and one mitochondrial (COI). All the fragments were amplified and sequenced, according to the protocols described by STEC *et al.* (2020b). The primers with the original references are listed in Table 1. The sequencing products were read with the ABI 3130xl sequencer at the Department of Biological and Environmental Science of the University of Jyväskylä (Jyväskylä, Finland). The

Table 1

PCR primers for amplification of the four DNA fragments sequenced in the study

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source
18S rRNA	18S_Tar_1Ff	Forward	AGGCGAAACCGCGAATGGCTC	STEC *et al.* (2017)
	18S_Tar_1Rr	Reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28S_Eutar_F	Forward	ACCCGCTGAACTTAAGCATAT	GĄSIOREK *et al.* (2018), MIRONOV *et al.* (2012)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC	
ITS-2	Eutar_Ff	Forward	CGTAACGTGAATTGCAGGAC	STEC *et al.* (2018)
	Eutar_Rr	Reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	FOLMER *et al.* (1994)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	

Table 2

GenBank accession numbers of sequences used in the phylogenetic analysis

	18S	28S	COI	ITS2	Source
Crenubiotus salishani **sp. nov.** T1	ON062322	ON062305	ON059359	ON062326	This study
Crenubiotus salishani **sp. nov.** T2	ON062323	ON062306	ON059360	ON062327	This study
Crenubiotus crenulatus NO.429	MT812474	MT812468	MT808079	MT812606	STEC *et al.* (2020a)
Crenubiotus sp. GL.001 1	OM179850	OM179857	OM151284	OM179864	STEC & MOREK 2022
Crenubiotus sp. GL.001 2	OM179851	OM179858	OM151285	OM179865	STEC & MOREK 2022
Crenubiotus ruhesteini V6	MW074387		MW074338	MW074370	GUIDETTI *et al.* 2021
Crenubiotus ruhesteini T3	MW074386			MW074369	GUIDETTI *et al.* 2021
Crenubiotus ruhesteini T2	MW074385		MW074337	MW074368	GUIDETTI *et al.* 2021
Crenubiotus ruhesteini T1	MW074384		MW074336	MW074367	GUIDETTI *et al.* 2021
Crenubiotus ruhesteini V3	MW074389			MW074372	GUIDETTI *et al.* 2021
Crenubiotus ruhesteini V1	MW074388			MW074371	GUIDETTI *et al.* 2021
Crenubiotus sp. GB.108 1	MT812473	MT812467	MT808077	MT812604	STEC *et al.* (2020a)
Crenubiotus sp. GB.108 2	MT812473	MT812467	MT808078	MT812605	STEC *et al.* (2020a)
Adorybiotus sp. S1911 T1	ON062320	ON062303	ON059358	ON062324	This study
Adorybiotus sp. S1911 T2	ON062321	ON062304		ON062325	This study
Adorybiotus cf. *granulatus* JP.008 1	MT812475	MT812464	MT808075	MT812600	STEC *et al.* (2020a)
Adorybiotus cf. *granulatus* JP.008 2	MT812475	MT812464	MT808075	MT812601	STEC *et al.* (2020a)
Diaforobiotus islandicus IS.042	MT812470	MT812461	MT808072	MT812597	STEC *et al.* (2020a)
Diaforobiotus sp. NO.386	MT812471	MT812463	MT808074	MT812598	STEC *et al.* (2020a)
Richtersius tertius GR.008	MK211386	MK211384	MK214323	MK211380	STEC *et al.* (2020c), POGWIZD & STEC (2022)
Richtersius coronifer NO.385	MH681760	MH681757	MH676053	MH681763	STEC *et al.* (2020c)
Dactylobiotus selenicus	MT812476	MT812466	MT808076	MT812602	STEC *et al.* (2020a)
Murrayon cf. *pullari* IT.338	MT812477	MT812465	MT808080	MT812603	STEC *et al.* (2020a)

sequences were processed in MEGA7 (KUMAR *et al.* 2016) and were submitted to NCBI GenBank (Table 2).

Phylogenetic analysis

The phylogenetic analyses were performed using concatenated 18S rRNA+28S rRNA+ITS-2+COI sequences. All the *Crenubiotus* sequences available in GenBank were used, except for the 28S *C. ruhesteini* sequences from GUIDETTI *et al.* (2021) as those did not overlap with the sequences produced in this study. Additionally, representative sequences of *Adorybiotus*, Murrayidae and Richtersiusidae were included as outgroups (Table 2). The 18S rRNA, 28S rRNA and ITS-2 sequences were aligned with MAFFT ver. 7 (KATOH *et al.* 2002; KATOH & TOH 2008) with the G-INS-i method (thread=4, threadtb=5, threadit=0,

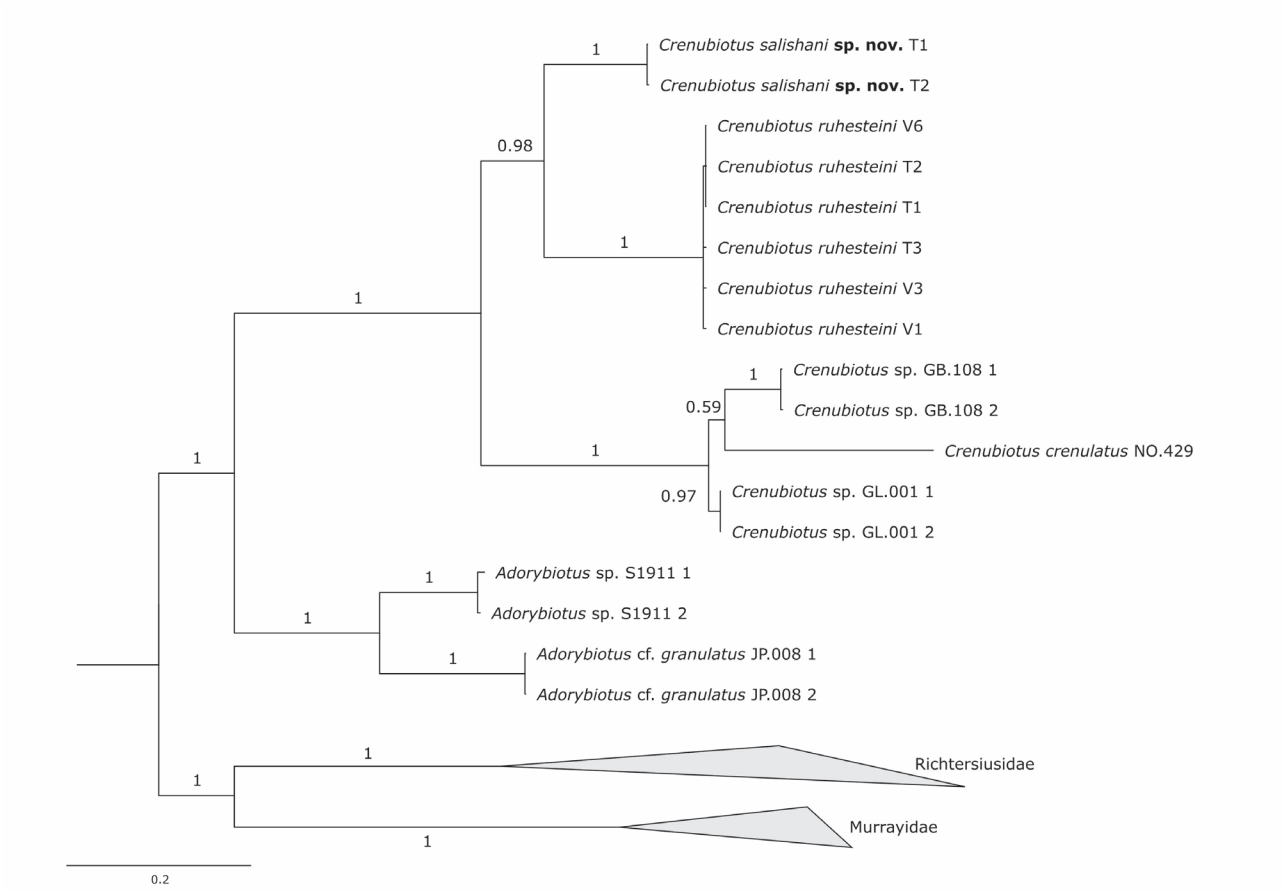

Fig. 1. Bayesian phylogenetic placement of the new *Crenubiotus* species. Values above/below the branches are Bayesian posterior probability values. The scale bar indicates mutations/sites.

reorder, adjustdirection, anysymbol, maxiterate=1000, retree 1, globalpair input). The ITS-2 sequences were also aligned using MAFFT ver. 7 with the G-INS-i method (thread=4, threadtb=5, threadit=0, reorder, adjust direction, any symbol, max iterate=1000, retree=1, global pair input). The COI sequences were aligned according to their amino acid sequences (translated using the invertebrate mitochondrial code) with the MUSCLE algorithm (EDGAR 2004) in MEGA7 with the default settings (all gap penalties=0, max iterations=8, clustering method=UPGMB, lambda=24). The alignments were visually inspected and trimmed in MEGA7. The sequences were concatenated with the R package 'concatipede' v1.0.0 (VECCHI & BRUNEAUX 2021). A model selection was performed for each alignment partition (6 in total: 18S rRNA, 28S rRNA, ITS-2 and three COI codons) with PartitionFinder2 (LANFEAR et al. 2016), with a partitions and models selection process, and the results are present in the Supplementary Materials (SM.02). The BI phylogenetic reconstruction was performed with MrBayes v3.2.6 (RONQUIST et al. 2012). Two runs with one cold chain and three heated chains were run for 20 million generations with a burning of 2 million generations, while sampling a tree every 1000 generations. The posterior distribution sanity was checked with Tracer v1.7 (RAMBAUT et al. 2018). The MrBayes input file with the input alignment is available as part of the Supplementary Materials (SM.03). The phylogenetic tree was visualised with FigTree v1.4.4 (RAMBAUT 2007) and the image was edited with Inkscape 0.92.3 (BAH 2011). The complete phylogenetic tree is available in SM.04.

Results

Phylogenetic reconstruction

The phylogenetic reconstruction (Fig. 1) recovered the family Adorybiotiidae as monophyletic. The newly-sequenced *Crenubiotus* individuals from Canada formed a monophyletic clade well separated from all the other *Crenubiotus* sequences present in GenBank.

Morphological analysis

The analysis of the morphological characteristics of the animals and eggs revealed that they do not belong to any currently described species. For a detailed account of those characteristics, see the Taxonomic account below.

Taxonomic account

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Parachela Schuster, Nelson, Grigarick & Christenberry, 1980

Superfamily: Macrobiotoidea Thulin, 1928 (in MARLEY *et al*. 2011)

Family: Adorybiotidae Stec, Vecchi & Michalczyk, 2020 (in STEC *et al*. 2020a)

Genus: *Crenubiotus* Lisi, Londoño & Quiroga, 2020

Crenubiotus salishani **sp. nov**. Vecchi, Choong & Calhim, 2022

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Tables 3-4; Figures 2-6; Supplementary Material SM.01

Materials examined: Six animals and 14 eggs; specimens were mounted on microscope slides in Hoyer's medium (4 animals + 14 eggs) and processed for DNA sequencing (2+0).

Type of locality: 48°46'16.8"N 123°22'39.7"W, Salt Spring Island in British Columbia, Canada; moss on rock; coll. 24th of June 2021 by Henry Choong; the new species was found together with *Macrobiotus* sp.

Type of repository: Holotype (JYUt.S1916_SL2_B), 3 paratypes (slides JYUt.S1916_SL1 and S1916_SL_2: RBCM 022-00002-001) and 14 eggs (slides JYUt.S1916_SL1 and S1916_SL_3: RBCM 022-00002-002) were deposited in the Natural History Collections of the Jyväskylä University Museum, Ihantolantie 5, Jyväskylä, Finland (JYU) and in the collections of the Invertebrate Zoology Department, Royal BC Museum (RBCM), 675 Belleville Street, Victoria, BC Canada, V8W 9W2.

Etymology: The species name refers to the Salish Sea, where Salt Spring Island is located and where the new species was found, in recognition of the islands in the Salish Sea as places of special concern for biological diversity.

Species description

Animals (measurements and statistics are included in Table 3)

The body is whitish-transparent; after fixation in Hoyer's medium, the body is transparent (Figure 2A). Eyes are not visible in the mounted specimens. Body cuticles with elliptical-circular (0.53-0.73 μm; SM.01) and quadrangular bigger pores (1.01-1.53 μm; SM.01) are distributed randomly on the entire body cuticle, with the biggest quadrangular pores being present on the dorso-caudal body region (Figure 2B; Table 4). Patches of dense granulation are present on the dorso-caudal end of the body (Figure 2C). Patches of dense granulation are present on all legs (Figures 2D-E). On Legs I-III, the dense granulation comprises a continuous band that starts on the external leg surface, extends into the frontal leg surface and ends on the internal leg surface (Figures 2D-E). On Legs IV, the dense granulation evenly covers the dorsal and both lateral leg surfaces (Figure 3A). Claws are slender and of the Richtersiusidae type. There are primary branches with distinct accessory points, a long, constricted in the middle, common tract, and with an evident stalk connecting the claw to the lunula and a system of internal septa (Figures 3B-C). The system consists of a laminar peduncle with a distally enlarged portion, and a complete septum separating it to an intermediate tract (Figure 3B). A suture in the distal common tract following the complete septum is visible (Figure 3C). Big, trapezoidal lunulae, with long and evenly distributed teeth are present on all legs (Figures 3B-C). Paired muscle attachments present just below the lunulae are faintly visible (Figure 2E). The mouth is antero-ventral. There is a buccopharyngeal apparatus of a modified '*Macrobiotus* type' (Figure 4A), i.e. with a rigid buccal tube with ventral lamina. There is a pharyngeal bulb with triangular apophyses (Figures 4B-C), three anterior cuticular spikes on the anterior end of the placoids row (typically, only two are visible in any given plane, Figure 4C) and two rod-shaped macroplacoids and a microplacoid positioned close to the second macroplacoid (Figures 4B-C). The first macroplacoid is longer than the second. The first macroplacoid is also anteriorly narrowed and constricted in the middle, whereas the second has a sub-terminal constriction (Figures 4B-C). The oral cavity armature is poorly developed and is composed only of the third band of teeth (Figures 4D-F). The first and the second band of teeth are absent or invisible under PCM (Figures 4D-F). The teeth of the third band are located within the posterior portion of the oral cavity, anteriorly to the buccal tube opening (Figures 4D-F). The third band of teeth is divided into the dorsal and the ventral portions. Under PCM, both the dorsal and the ventral portions are seen as two distinct transverse ridges, and the dorsal ones form a globular thickening at the me-

Table 3

Measurements (in μm) of selected morphological structures of the animals of *Crenubiotus salishani* **sp. nov.** mounted in Hoyer's medium (N = number of individuals/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation)

Character	N	Range		Mean		SD		Holotype	
		μm	*pt*	μm	*pt*	μm	*pt*	μm	*pt*
Body length	4	319 - 350	*951 - 1053*	334	*1004*	13	*52*	350	*1053*
Buccopharyngeal tube									
Buccal tube length	4	31.9 - 34.4		33.3		1.0		33.2	
Stylet support insertion point	4	23.8 - 26.2	*73.9 - 76.2*	25.0	*75.1*	1.0	*1.0*	25.1	*75.5*
Buccal tube external width	4	3.5 - 3.8	*10.0 - 11.4*	3.6	*10.8*	0.2	*0.6*	3.8	*11.4*
Buccal tube internal width	4	2.1 - 2.3	*6.1 - 7.0*	2.1	*6.4*	0.1	*0.4*	2.1	*6.3*
Ventral lamina length	3	15.0 - 16.9	*45.1 - 50.5*	16.0	*47.3*	1.0	*2.8*	15.0	*45.1*
Placoid lengths									
Macroplacoid 1	4	6.8 - 8.9	*19.8 - 27.9*	7.5	*22.7*	1.0	*3.7*	7.5	*22.4*
Macroplacoid 2	4	5.3 - 7.1	*15.5 - 21.1*	6.3	*19.0*	0.7	*2.5*	6.6	*19.8*
Microplacoid	4	1.3 - 2.3	*4.2 - 6.8*	1.9	*5.7*	0.5	*1.2*	1.7	*5.2*
Macroplacoid row	4	13.7 - 16.1	*39.7 - 50.4*	14.9	*45.0*	1.0	*4.4*	14.8	*44.6*
Placoid row	4	16.3 - 18.7	*47.5 - 58.7*	17.5	*52.6*	1.1	*4.7*	16.9	*50.9*
Claw I heights									
External base	0								
External primary branch	4	6.4 - 8.1	*19.3 - 25.2*	7.2	*21.7*	0.7	*2.6*	6.4	*19.3*
External secondary branch	4	5.3 - 6.8	*15.5 - 21.3*	6.0	*18.1*	0.8	*2.8*	5.3	*16.0*
External base/primary branch (cct)	0			–		–		–	
Internal base	2	3.6 - 4.7	*11.4 - 14.1*	4.2	*12.7*	0.8	*1.9*	–	–
Internal primary branch	4	5.8 - 8.0	*16.8 - 24.9*	6.8	*20.5*	1.0	*3.5*	6.2	*18.8*
Internal secondary branch	2	6.2 - 6.4	*18.5 - 20.2*	6.3	*19.3*	0.2	*1.2*	–	–
Internal base/primary branch (cct)	2	45.6 - 65.5		55.6		14.1		–	
Claw II heights									
External base	3	3.8 - 4.9	*11.9 - 14.7*	4.5	*13.7*	0.6	*1.5*	4.8	*14.4*
External primary branch	4	7.0 - 8.2	*20.5 - 24.7*	7.5	*22.7*	0.6	*2.3*	7.0	*20.9*
External secondary branch	4	4.9 - 7.0	*14.3 - 20.7*	6.0	*18.1*	0.9	*3.0*	5.6	*16.8*
External base/primary branch (cct)	3	48.2 - 68.7		59.0		10.3		68.7	
Internal base	3	4.4 - 5.0	*13.7 - 14.9*	4.8	*14.4*	0.3	*0.6*	4.9	*14.6*
Internal primary branch	4	6.5 - 8.9	*18.9 - 26.7*	7.6	*22.7*	1.0	*3.2*	8.9	*26.7*
Internal secondary branch	4	5.2 - 6.4	*15.7 - 19.0*	5.9	*17.6*	0.5	*1.5*	5.2	*15.7*
Internal base/primary branch (cct)	3	54.9 - 64.6		60.5		5.1		54.9	
Claw III heights									
External base	4	4.6 - 5.8	*13.3 - 17.2*	5.3	*15.9*	0.6	*1.8*	5.7	*17.2*
External primary branch	4	7.7 - 8.3	*22.5 - 26.1*	8.0	*24.1*	0.3	*1.5*	7.9	*23.8*
External secondary branch	4	6.4 - 6.6	*18.7 - 20.0*	6.5	*19.5*	0.1	*0.6*	6.6	*19.9*
External base/primary branch (cct)	4	59.2 - 72.3		66.0		6.7		72.3	
Internal base	3	4.3 - 5.2	*12.6 - 16.2*	4.6	*13.9*	0.5	*2.0*	4.3	*13.0*
Internal primary branch	4	7.5 - 8.0	*21.8 - 25.0*	7.8	*23.4*	0.2	*1.3*	7.8	*23.6*
Internal secondary branch	4	5.7 - 6.4	*17.0 - 19.3*	6.0	*18.2*	0.3	*1.2*	6.4	*19.3*
Internal base/primary branch (cct)	3	55.0 - 64.8		59.3		5.0		55.0	
Claw IV heights									
Anterior base	3	4.9 - 5.5	*14.7 - 17.4*	5.2	*15.7*	0.3	*1.5*	4.9	*14.7*
Anterior primary branch	4	8.5 - 9.4	*25.3 - 29.3*	9.0	*27.0*	0.4	*1.8*	8.5	*25.7*
Anterior secondary branch	4	6.7 - 7.5	*19.4 - 22.6*	7.2	*21.6*	0.4	*1.5*	7.5	*22.6*
Anterior base/primary branch (cct)	3	54.9 - 59.2		57.0		2.2		57.0	
Posterior base	3	4.7 - 5.8	*14.0 - 17.2*	5.2	*15.9*	0.6	*1.6*	4.7	*14.0*
Posterior primary branch	3	8.4 - 9.2	*25.4 - 28.9*	8.9	*26.9*	0.4	*1.8*	8.4	*25.4*
Posterior secondary branch	3	6.7 - 7.6	*20.1 - 23.7*	7.1	*21.5*	0.5	*2.0*	6.7	*20.1*
Posterior base/primary branch (cct)	3	55.3 - 64.6		58.9	–	5.0		55.3	

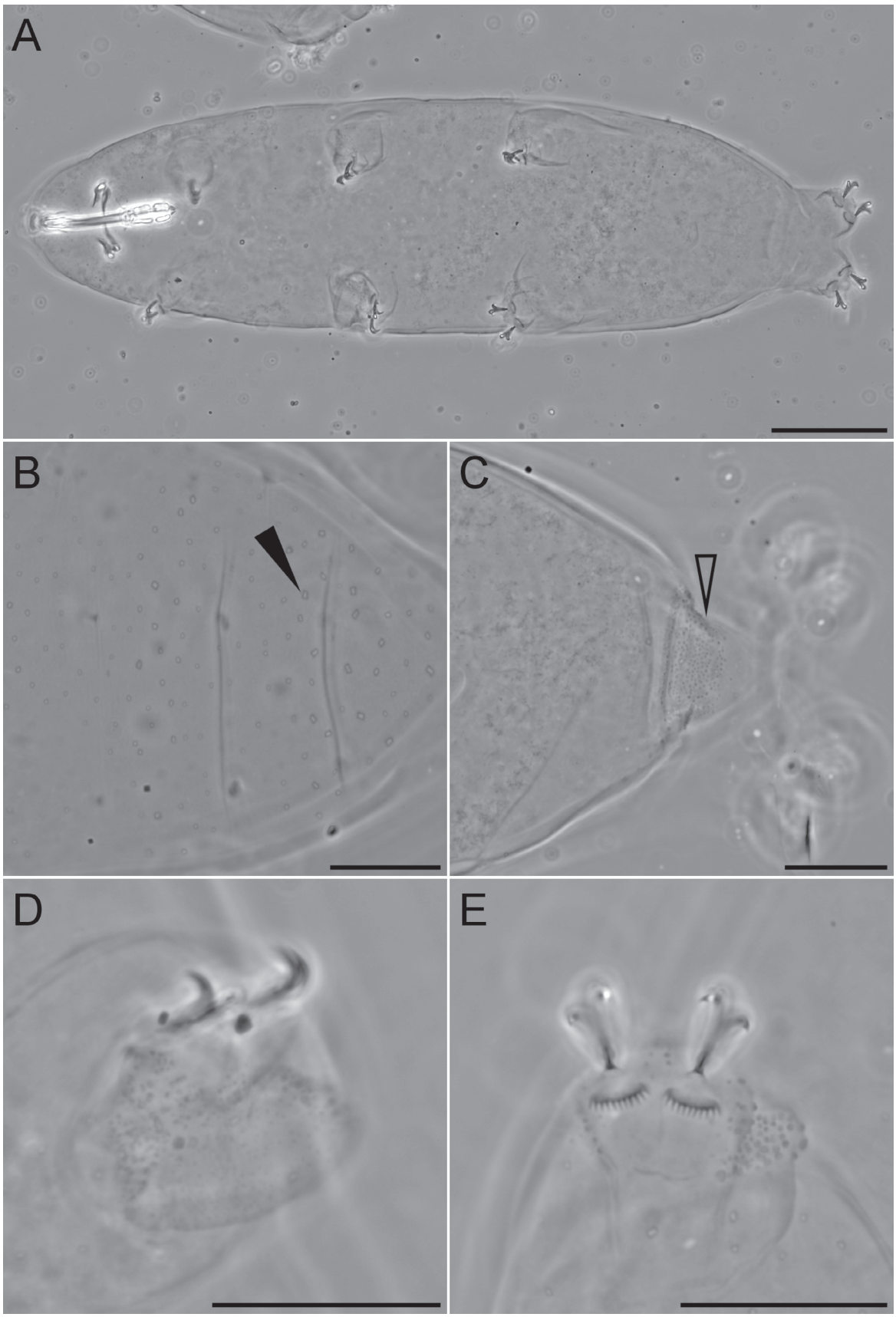

Fig. 2. *Crenubiotus salishani* sp. nov. habitus and cuticle granulations under PCM. A – Habitus of holotype; B – Cuticular pores present in the dorso-caudal part of the body; C – Dorso-caudal band of granulation; D – Leg III external granulation; E – Leg III external and internal granulation. A filled arrowhead indicates quadrangular pores and an empty arrowhead indicates the dorso-caudal band of granulation. Scale bars: A 50 µm, B-E 20 µm. A, C, D: Holotype S1916_SL2_B RBCM 022-00002-001; B: Paratype JYUt.S1916_SL1_A.

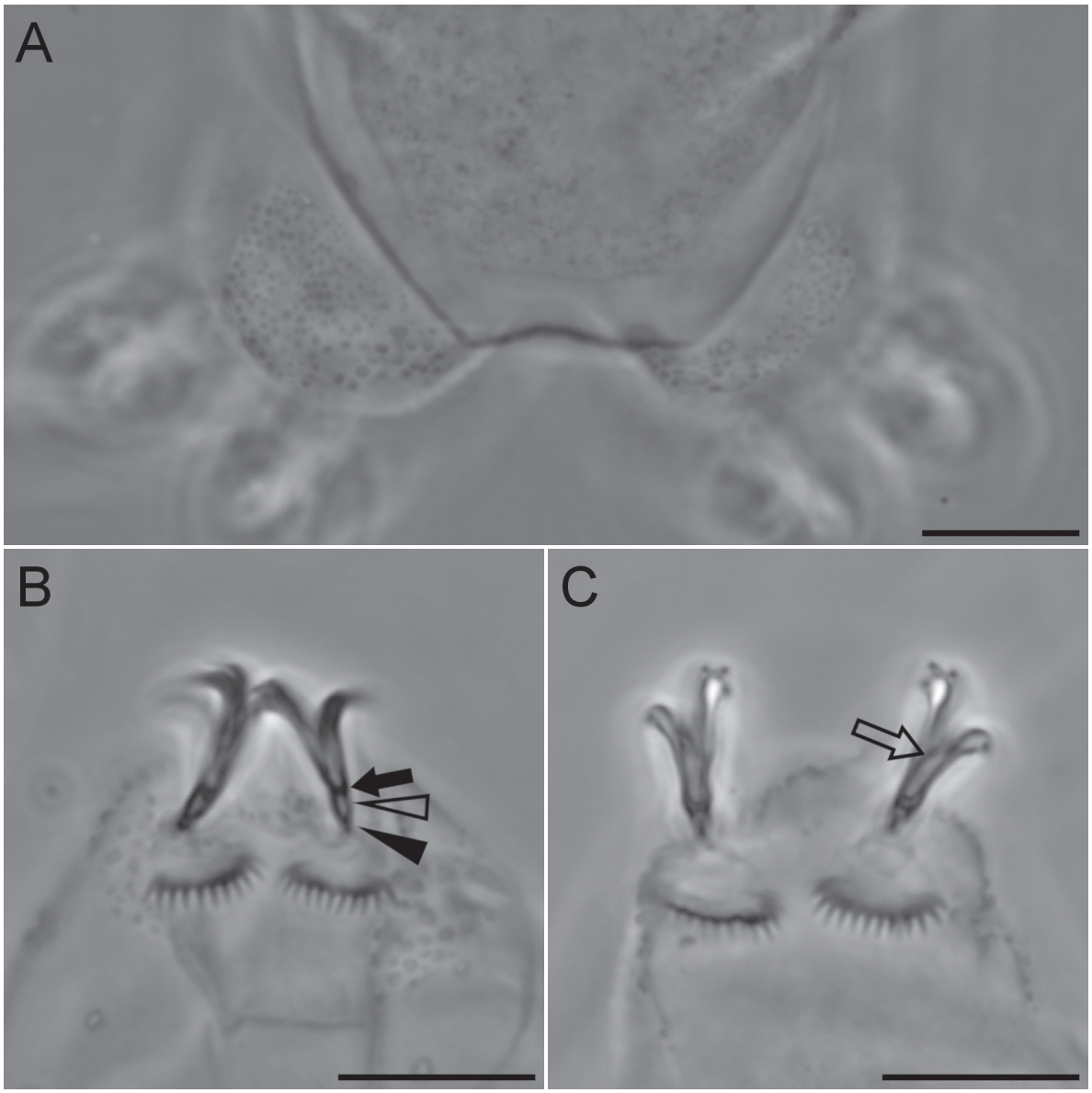

Fig. 3. *Crenubiotus salishani* sp. nov. Leg IV granulation and claws under PCM. A – Leg IV dorsal granulation; B – Claws and lunules on Leg III; C – Claws and lunules on Leg IV. An arrowhead indicates the basal portion of the stalk system, an empty arrowhead indicates the distal enlarged portion of the stalk system, an arrow indicates the complete septum, and an empty arrow indicates the suture in the distal common tract. Scale bar: 10 µm. A, C: Holotype S1916_SL2_B RBCM 022-00002-001; B: Paratype JYUt.S1916_SL1_A.

dial extremity (Figure 4D-E). A proper median tooth is absent on both sides (dorsal and ventral) of the third band of teeth; however, a single median mucrone is present anteriorly to the dorsal ridges. (Figure 4D-E).

Eggs (measurements and statistics are included in Table 4)

The eggs are aid freely, whitish, spherical with conical processes, with elongated apices and an egg surface without areolation (Figs 5A, 6). Irregular distributed thickenings on the chorion, seen in PCM as striae connecting neighbouring processes, are present (Figure 5A-B) but are not visible in CF (Figure 6). Apart from these structures, the egg surface between processes appears smooth under PCM (Figure 5B). The elongated and flexible process apices are rarely multifurcated (Figure 5C), with a faint granulation on them visible in PCM (Figure 5C-D). There is no visible labyrinthine layer between the process walls (Figures 5C-D). Small thickenings around the process bases are present; however, they are very faintly visible in PCM (Figure 5B).

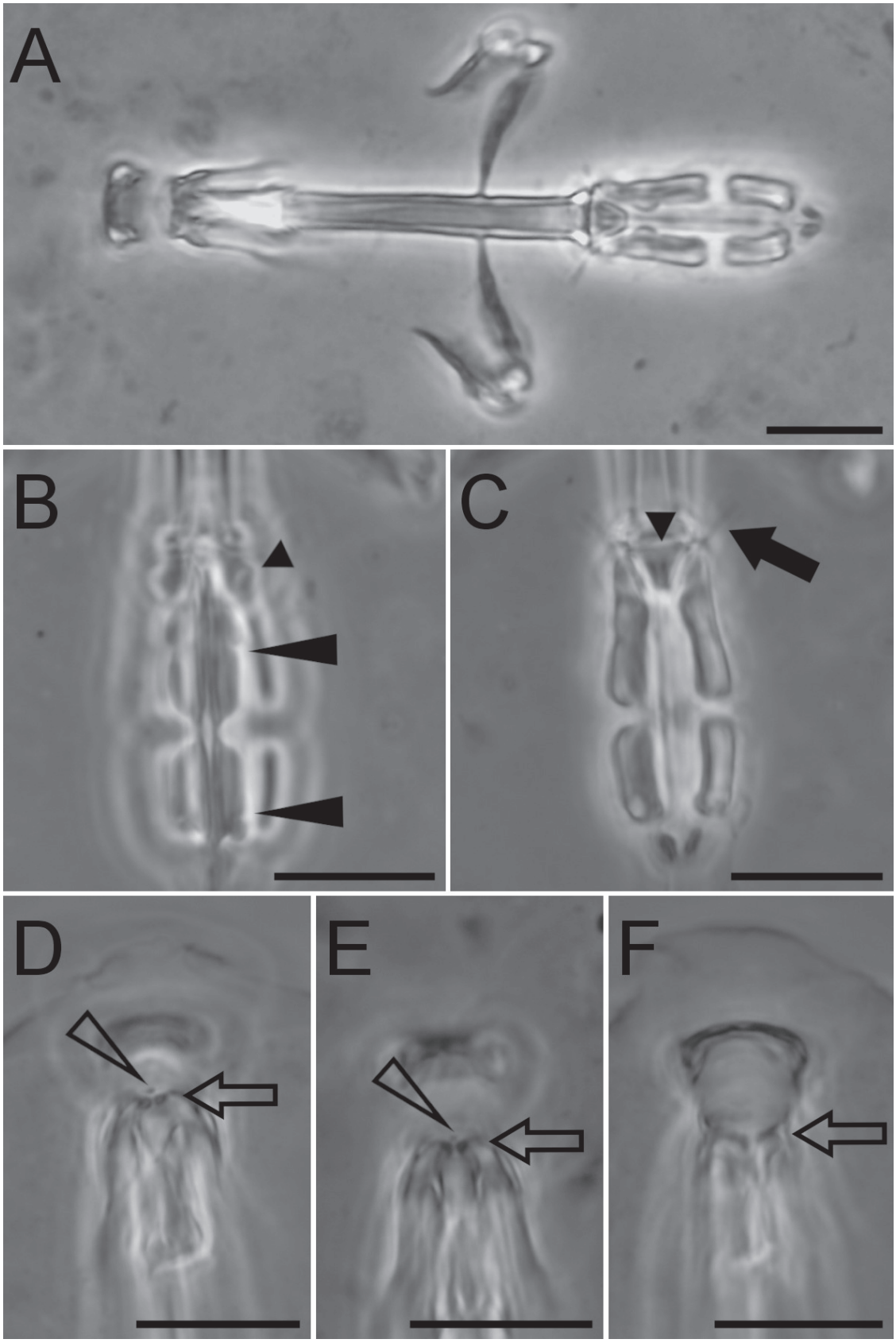

Fig. 4. *Crenubiotus salishan*i sp. nov. buccal apparatus under PCM. A – Buccal apparatus *in toto*; B – Dorsal placoids; C – Ventral placoids; D-E – Dorsal portion of the oral cavity armature (OCA); F – Ventral portion of the OCA. Filled arrowheads indicate constrictions on the macroplacoids, a filled arrow indicates a dorsal cuticular spike, triangles indicate triangular apophyses in the buccal apparatus, empty arrows indicate the third band of the OCA, and empty arrowheads indicate anterior median mucrone in the third band of the OCA. Scale bar: 10 µm. A, E: Paratype JYUt.S1916_SL1_A; B, C: Paratype S1916_SL2_A RBCM 022-00002-001; D, F: Holotype S1916_SL2_B RBCM 022-00002-001.

Table 4

Measurements (in μm) of selected morphological structures of the eggs of *Crenubiotus salishani* **sp. nov.** mounted in Hoyer's medium (N = number of eggs/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation)

Character	**N**	Range	Mean	SD
Egg bare diameter	14	48.0 - 59.2	54.0	2.6
Egg full diameter	14	65.0 - 75.7	70.6	2.8
Process height	42	4.7 - 10.0	7.4	1.1
Process base width	42	3.6 - 7.8	6.1	0.9
Process base/height ratio	42	46% - 126%	84%	17%
Inter-process distance	42	1.9 - 5.0	3.1	0.6

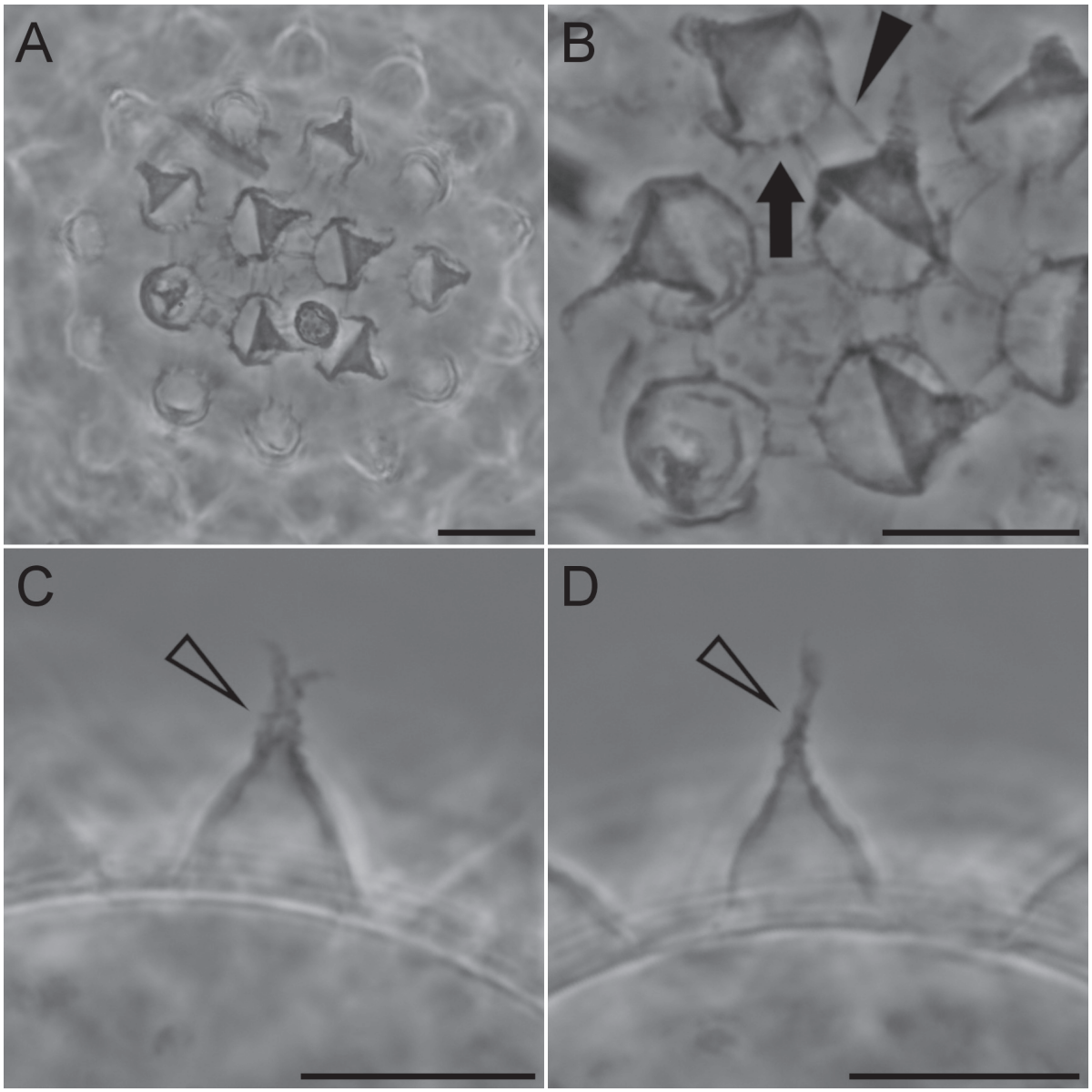

Fig. 5. *Crenubiotus salishani* sp. nov. eggs under PCM. A – Eggshell overview; B – Chorion and processes details; C – Egg process with a bifurcating tip in section; D – Egg process with a single tip in section. An arrowhead indicates a stria connecting two processes, empty arrowheads indicate granulation on the tips of the processes, and an arrow indicates thickenings around the process base. Scale bar: 10 μm. A, B, C, D: Paratype S1916_SL3 RBCM 022-00002-002.

Reproduction

Unknown.

DNA sequences

18S: ON062322, ON062323
28S: ON062305, ON062306
COI: ON059359, ON059360
ITS2: ON062326, ON062327

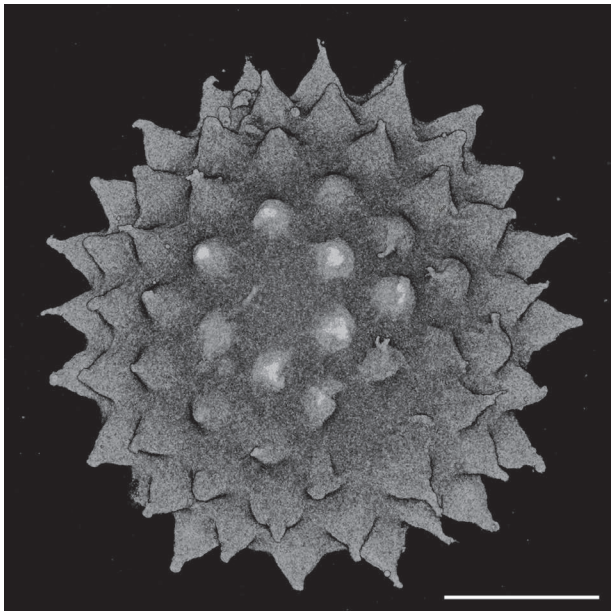

Fig. 6. *Crenubiotus salishani* sp. nov. egg 3D reconstruction with CF. Scale bar: 20 µm. Paratype S1916_SL3 RBCM 022-00002-002.

Discussion

Differential diagnosis

The new species differs from the other three species in the genus by the absence of a visible labyrinthine layer in the egg processes. In addition, it also differs from its congeners in the following manner:

– From *C. crenulatus* due to: the presence of large quadrangular pores (only roundish pores are present in *C. crenulatus*), a shorter ventral lamina (19.8-25.3 µm (*pt 53.4-61.7*) in C. *crenulatus* vs. 15.0-16.9 µm (*pt 45.1-50.5*) in C. *salishani* sp. nov.), and by less evident tubercles within the dorso-caudal bend.

– From *C. revelator* due to: the presence of large quadrangular pores (only roundish pores are present in *C. revelator*), less evident tubercles within the dorso-caudal bend, and by the presence of a medio-dorsal mucrone in the buccal armature (the mucrone is medio-ventral in *C. revelator*).

– From *C. ruhesteini* due to: the lack of a large pore on the legs, slightly larger pt values for the primary branches of anterior claws on the fourth pair of legs (*pt 18.825.3* in *C. ruhesteini* vs. *25.3-29.3* in *C. salishani* sp. nov.), and by less evident tubercles within the dorso-caudal bend.

Both the molecular and morphological analysis point to the uniqueness of the newly found population of *Crenubiotus* and support its status as a distinct and new species, which increases the number of species in the genus from 3 to 4. The number of described species in the genus with associated DNA sequences also increases from 3 to 4 (*C. crenulatus*, *C. ruhesteini* and *C. salishani*). However, as is shown by the tree in Figure 1, sequences from two more species (*Crenubiotus* sp. GB.108 from the United Kingdom and *Crenubiotus* sp. GL.001 from Greenland) are present. As it is not possible to determine at this point if one of those two undetermined species corresponds to *C. revelator*, those sequences could belong to one (if one of them belongs to *C. revelator*) or two additional undescribed species.

KATHMAN (1990) reported on *Macrobiotus echinogenitus* from British Columbia (Flower Ridge, Vancouver Island) and provided a detailed illustration and description of the specimens (KATHMAN 1990, p. 1889-1890, Fig. 16 A-G). It is now clear that the individuals found in that study can be confidently classified as *Crenubiotus* sp. The description of the individuals found by KATHMAN (1990) does not agree with the animals found in this study (see the Taxonomic account section for a detailed description) in relation to two characteristics: i) the presence of eyes (not observed in the freshly-mounted animals from Salt Spring Island); and ii) the smooth egg chorion (with evident thickenings on the eggshell connecting the processes in the eggs from Salt Spring Island). Given the difference in eggshell morphology between Katzman's specimens and the individuals described in the current study, it is possible that the *Crenubiotus* population found by KATHMAN (1990) represents a different species than the one found on Salt Spring Island, even if the possibility that the eyes were dissolved by the mounting medium cannot be excluded, as occurred in the study by POGWIZD & STEC (2020).

The discovery of *Crenubiotus salishani* on Salt Spring Island provides additional data regarding the occurrence and distribution of tardigrades in British Columbia and their ecology. To date, relatively few studies have dealt with the ecological implications of tardigrade distributions and the elevation, species of mosses or different mountains (KATHMAN 1990). Although there may be little or no evidence to support the relationship between tardigrade distributions and these variables on a larger scale, microhabitat factors

may determine their distribution and density (KATHMAN & CROSS 1991). Furthermore, biodiversity discoveries continue to be important in the Salish Sea region, which extends across Salishan and other Indigenous lands, as well as the border between Canada and the United States, especially in the context of the accelerated convergence of local and global environmental stressors, and the cumulative impacts of long-term development and the alteration of these watersheds and seascapes (SOBOCINSKI 2021).

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Author Contributions

Research concept and design: M.V., H.C., S.C.; Collection and/or assembly of data: M.V.; Data analysis and interpretation: M.V., H.C., S.C.; Writing the article: M.V., H.C., S.C.; Critical revision of the article: M.V., H.C., S.C.; Final approval of article: M.V., H.C., S.C.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary Material to this article can be found online at:

http://www.isez.pan.krakow.pl/en/folia-biologica.html

Supplementary files:

SM.01. Morphometric data for *Crenubiotus salishani* **sp. nov.**

SM.02. Model selection results on the concatenated alignment.

SM.03. MrBayes input file for the phylogenetic analysis, including the used alignment.

SM.04. Output phylogenetic tree from the MrBayes analysis.

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